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Production of itaconic acid by *Pseudozyma antarctica* NRRL Y-7808 under nitrogen-limited growth conditions

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Abstract

Pseudozyma antarctica NRRL Y-7808 was found to produce itaconic acid from glucose and other sugars under nitrogen-limited growth conditions. Other *Pseudozyma* strains screened, including a second strain of *P. antarctica*, did not produce this product; therefore itaconic acid production is not a common trait of the genus. Glucose and fructose were the most efficiently utilized substrates, followed by sucrose and maltose; lactose and glycerol were the poorest substrates. The maximum yield in flask experiments was 37.5% (approximately 30 g/L itaconic acid from 80 g/L glucose). The maximum rate of production in flask cultures was 248 mg L^{-1} h^{-1} at a C/N ratio of 116, while the best combination of rate and yield was produced at a C/N ratio of 175 (230 mg L^{-1} h^{-1} and 29%, respectively). A stirred-tank reactor process study resulted in a 20% yield and volumetric production rate of 110 mg L^{-1} h^{-1}.
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Keywords: Itaconic acid; Nitrogen limitation; *Pseudozyma antarctica* NRRL Y-7808; Sugar utilization; Bioreactor

1. Introduction

Itaconic acid (IA) (CAS #: 97-65-4) (methylenebutanedioic acid) is an α-substituted acrylic acid that is used in the manufacture of synthetic resins, coatings, and other industrial products [1,2]. It is produced commercially by the fungal fermentation of carbohydrates [1,2]. The total market for IA has been quoted as being between 10,000 and 15,000 metric tonnes per year worldwide [3] with a price that is 10-times that of citric acid, a more widely used fermentation product [4].

The organism most often used for IA production is *Aspergillus terreus*, grown under phosphate-limited conditions [2,5,6], although some species of the plant pathogenic fungal genus *Ustilago*, a basiodiomycete, are also known to produce IA during fermentation [2]. The sensitivity of *A. terreus* fermentations to metal concentrations [6] and difficulties working with filamentous organisms in bioreactors has led to the testing of yeasts for possible IA production. The patent literature in this area, reviewed by Willke and Vorlop [2], includes reports of IA production by a *Candida* mutant strain and *Rhodotorula* species. Tabuchi et al. [7] isolated a strain, putatively identified as a *Candida*, that produced IA at a 35% yield when grown under phosphate-limited conditions. Here we report on the ability of *Pseudozyma antarctica* NRRL Y-7808 to produce itaconic acid from glucose and other sugars under nitrogen-limited growth conditions. Species of *Pseudozyma* are basidiomycetes and are believed to be closely related to *Ustilago* [8].

2. Materials and methods

Yeast strains screened were from the ARS culture collection (NRRL), National Center for Agricultural Utilization Research, Peoria, Illinois, and were maintained on potato-dextrose-agar slants throughout this study. Itaconic acid (>99%) and thiamine hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO). Trypticase-soy broth (TSB), dextrose (glucose), and yeast extract were products of Becton, Dickinson and Co. (Sparks, MD). Other medium components were laboratory grade or better and used without purification.

Initial screening for organic acid production under nitrogen-limited growth conditions was carried out in a medium with the following composition (g/L): Glucose or glycerol, 80; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 1.7; Na_2HPO_4, 12; $MgSO_4{\cdot}7H_2O$, 1.4; $CaCl_2$, 0.02; $ZnSO_4{\cdot}7H_2O$, 0.02; $FeSO_4{\cdot}7H_2O$, 0.05; $MnSO_4{\cdot}H_2O$, 0.02; thiamine hydrochloride, 0.006; yeast extract, 0.5. Initial pH was 6.0 and sterile bromocresol purple (32 mg/L) was added post-autoclave as a pH indicator. After initial screening, the following modifications were made to the medium for itaconic acid production test experiments: $MgSO_4{\cdot}7H_2O$, 2.5 g/L; $ZnSO_4{\cdot}7H_2O$, 1.5 μg/L; $MnSO_4{\cdot}H_2O$, 0.9 μg/L. Other medium modifications made during these experiments are noted below.

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The medium was inoculated (1%) from 48-h cultures grown on TSB. Initial screening was done in 50 mL Erlenmeyer flasks containing 15 mL medium. Itaconic acid production experiments were either 25 or 50 mL volumes in 125-mL flasks. Growth conditions were 28 °C and 200 rpm. During growth, a pH indicator was used to maintain the pH within a desired range by daily additions of 2 M KOH. Growth in the screening experiment was continued until all flasks stopped requiring base addition (day 11). Using pH change as a guide, itaconic acid production experiments were incubated for ten days. Initial screening was done in single cultures, while IA production studies were performed in duplicate.

A fermentation experiment was performed in an Applikon fermentor controlled by an ADI 1030 Biocontroller equipped with sensors for monitoring temperature, pH, and dissolved oxygen, and monitored with BioXpert software (Applikon, Inc., Foster City, CA). The 2 L dished-bottom reactor contained 1 L of medium. Air was supplied at 1 vvm with agitation provided by two marine impellers at 1000 rpm. Temperature was maintained at 28 °C. The fermentor was equilibrated at these conditions for 1 h prior to inoculation and the oxygen concentration was set to a nominal 100% by calibrating the oxygen probe. After equilibration, the medium was inoculated with 10 mL (1%) of a 24-h-old TSB culture. Initial pH of the medium was 6.0 and was allowed to drop to 5.0 where it was held steady by the addition of 2 M KOH. Antifoam SO-25 (Sigma–Aldrich, St. Louis, MO) was added as needed. Duplicate samples were taken at each time point.

Analysis of culture supernatants for organic acids was performed by HPLC with a Phenomenex Synergi Fusion-RP column (150 mm × 4.6 mm, 4 μm particle size). The mobile-phase was 0.25% acetic acid (isocratic) and detection was at 201 nm with a diode-array detector. Culture supernatants from the screening experiment that exhibited possible organic acid production were acidified and extracted with ethyl acetate. The extracts were methylated with diazomethane and analyzed by GC–MS.

Sugar concentrations were assayed by the anthrone/sulfuric acid method. Samples were diluted in 50 mM sodium bicarbonate buffer. One volume sample was placed in vials and cooled to 4 °C prior to addition of 2.5 volumes of the anthrone reagent (2 g/L anthrone in concentrated sulfuric acid). The vials were sealed, mixed, and heated to 95 °C for 15 min. After cooling, readings were taken at 625 nm.

3. Results and discussion

Fourteen yeast strains that had not previously been characterized for organic acid production, including eight from the genus *Pseudozyma*, were screened for organic acid biosynthesis using the nitrogen-limited screening medium described (C/N ratio = 352) (Table 1). Most of the strains tested did not grow well or did not produce significant amounts of acids when grown on glycerol. However, when the strains were grown on glucose, *P. antarctica* NRRL Y-7808 was found to require the most base addition in order to maintain the pH of the culture (data not shown). Its culture supernatant showed the production of a substantial amount of an unknown compound when analyzed by HPLC. This strain was selected for further work. A second *P. antarctica* strain (NRRL Y-8295) did not exhibit significant acid production from glucose. Mass spectral analysis of the screening culture extract of *P. antarctica* NRRL Y-7808 revealed the major compound to be itaconic acid as identified by a mass spectral library. The HPLC retention time of authentic itaconic acid was the same as the culture product.

Table 1
Strains screened for organic acid production

NRRL accession #	Species
YB-4297	*Aciculoconidium aculeatum*
YB-4298	*Aciculoconidium aculeatum*
YB-2364	*Candida bentonensis*
Y-5579	*Candida hispaniensis*
Y-5580	*Candida hispaniensis*
Y-7808	*Pseudozyma antarctica*
Y-8295	*Pseudozyma antarctica*
Y-7954	*Pseudozyma aphidis*
Y-17627	*Pseudozyma flocculosa*
Y-17173	*Pseudozyma fusiformata*
Y-27503	*Pseudozyma prolifica*
Y-17626	*Pseudozyma rugulosa*
Y-7792	*Pseudozyma tsukubaensis*
Y-1095	*Yarrowia lipolytica*

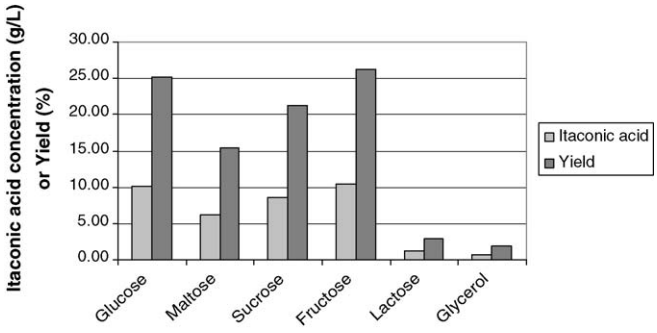

Fig. 1. Itaconic acid production by *Pseudozyma antarctica* NRRL Y-7808 from various carbon sources. Data are the average of duplicates. Yield is based on initial concentration of carbon source (40 g/L).

The initial screening medium was modified by dropping the concentrations of $FeSO_4$, $ZnSO_4$, and $MnSO_4$ to 0.005 mM for all from 0.18, 0.07, and 0.12 mM, respectively. This new medium was used as a base to test for the effects of magnesium, iron, calcium, and nitrogen concentration on the yield of itaconic acid in flask experiments. No effect on itaconic acid yield was seen at magnesium concentrations of 10 and 20 mM or iron concentrations between 0.01 and 1 mM, either with or without $CaCl_2$ at 0.2 mM. All cultures, including those grown on the initial screening medium, produced approximately 30 g/L itaconic acid from 80 g/L glucose in the starting medium (data not shown), a 37.5% yield. This yield is similar to that seen by Tabuchi et al. for a *Candida* strain grown under phosphate-limited conditions [7].

The ability of *P. antarctica* to utilize glycerol and sugars other than glucose to produce IA was also tested (Fig. 1). In this experiment, the carbon source concentration was 40 g/L and the nitrogen concentration was adjusted to maintain the C/N ratio used in the previous experiment. The monosaccharides glucose and fructose were utilized most efficiently, followed by the disaccharides sucrose and maltose. Lactose and glycerol were the poorest substrates, although both did yield slight amounts of IA (Fig. 1).

In experiments to determine the optimal nitrogen concentration, the magnesium and iron concentrations were set at 10 and 0.2 mM, respectively. Calcium chloride was omitted from the medium. Nitrogen concentrations between 0.038 and 45.6 mM [0.019–22.8 mM $(NH_4)_2SO_4$] were tested for initial rate and yield (Fig. 2). The initial rate was measured after the cultures started to produce itaconic acid, which was dependent on the initial nitrogen concentration (less nitrogen resulted in earlier

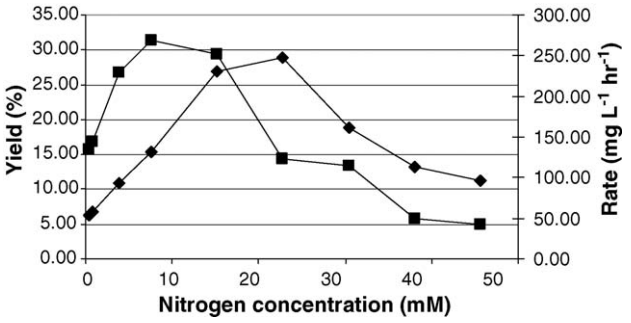

Fig. 2. Initial volumetric production rate and yield of itaconic acid vs. nitrogen concentration by *Pseudozyma antarctica* NRRL Y-7808. Diamond, rate; square, yield. Data are the average of duplicates. Yield is based on the initial glucose concentration (80 g/L).

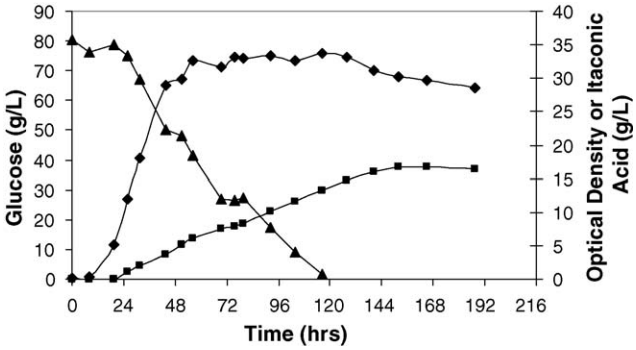

Fig. 3. Itaconic acid production by *Pseudozyma antarctica* Y-7808 in a fermentor with controlled pH. Triangle, glucose; diamond, optical density (A_{660}); square, itaconic acid. All data points are the average of duplicate samples.

induction of IA production). The initial volumetric production rate peaked at 248 mg L^{-1} h^{-1} with 22.8 mM nitrogen while the best yield (31%) was seen at 7.6 mM nitrogen. The best combination of rate and yield was achieved at 15.2 mM nitrogen with a rate of 230 mg L^{-1} h^{-1} and a yield of approximately 29%. The yield derived in this experiment was lower than that seen in previous experiments (for the same medium conditions). The reason for this variability is not known.

Due to the variable nature of the pH control in flask experiments, a fermentor-based experiment with direct pH control was undertaken. The medium used was that identified above as giving the best rate and yield (15.2 mM nitrogen) and pH indicator was not added for this experiment. Aeration was provided by 1 vvm sparger air and 1000 rpm agitation. These aeration conditions were nominally set as 100% oxygen prior to inoculation. After inoculation, the dissolved oxygen dropped to approximately 85% for a short period towards the beginning of the exponential growth phase (at ~20 h) and thereafter, rose slowly back to approximately 100%, where it remained for the balance of the fermentation, except for a brief interruption of aeration at approximately 72 h. The starting pH of the medium was 6.0 and fell with culture growth, reaching 5.0 at approximately 20 h of incubation time, and was held constant at that level for the rest of the fermentation.

There was an approximately 24-h lag time prior to the start of IA production, after which production proceeded at a linear rate of 132 mg L^{-1} h^{-1} until reaching a maximum concentration of 16.7 g/L at 152 h (or 6.3 days) (Fig. 3). This resulted in a yield of 20.9% or about 8% below that derived from flask studies with the same medium composition. The rate of production was below the 230 mg L^{-1} h^{-1} seen for the same medium composition in flask culture. The overall volumetric production rate, from inoculation to the point of maximum concentration was 110 mg L^{-1} h^{-1}. These yields and volumetric production rates were below the numbers quoted by Willke and Vorlop [2] as the maximum achieved in the *A. terreus* process, which has exhibited up to a ~47% yield and 1 g L^{-1} h^{-1} production rate.

Further fermentor studies may identify pH, temperature, dissolved oxygen concentrations, and cultivate densities more suitable for large-scale production of IA. The 24-h lag time prior to the beginning of production would likely be reduced by using a larger volume of inoculating culture. In this case, a 1% inoculation was used to reduce the addition of nutrients from the rich-medium used for the seed-culture. Utilization of a two-stage seed-culture protocol, with the second culture in the production medium, would remove this limitation.

4. Summary

Itaconic acid is commonly produced by *A. terreus* under phosphate-limited growth conditions. This type of growth-limitation was also used by Tabuchi et al. in a study of IA production in a strain of *Candida* [7]. Citric acid production in yeast strains, however, may be carried out under nitrogen-limited growth conditions [9,10] and this work shows that nitrogen-limitation is able to induce IA production in *P. antarctica* NRRL Y-7808. It is noteworthy that other *Pseudozyma* strains tested, including a second strain of *P. antarctica*, did not produce IA. Therefore, the ability to produce IA is a special property of *P. antarctica* NRRL Y-7808 and is not a common trait of the genus. Optimization of medium composition and fermentation parameters may result in improvements in production rate and yield over those exhibited in this study.

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